Novel Homozygous and Compound Heterozygous COL17A1 Mutations Associated with Junctional Epidermolysis Bullosa

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Junctional epidermolysis bullosa is a heritable, heterogeneous blistering skin disease with mechanically induced dermal–epidermal separation, mild skin atrophy, nail dystrophy, and alopecia. Four unrelated junctional epidermolysis bullosa families with different phenotypes were investigated here and four novel mutations associated with the disease were identified. Patients 1, 2, and 3 had generalized atrophic benign epidermolysis bullosa, with nonscarring blistering and varying degree of alopecia. Patient 4 had the *localisata* variant of junctional epidermolysis bullosa, with predominantly acral blistering and normal hair. All patients had mutations in the COL17A1 gene encoding collagen XVII, a hemidesmosomal transmembrane protein. Patients 1 and 2 carried homozygous deletions 520delAG and 2965delG, respectively. Patient 3 was compound heterozygous for a missense and a deletion mutation (G539E and 2666delTT), and patient 4 was heterozygous for a known mutation R1226X. The deletions led to premature termination codons and to drastically reduced collagen XVII mRNA and protein levels, consistent with the absence of the collagen in generalized atrophic benign epidermolysis bullosa skin. The missense mutation G539E allowed synthesis of immunoreactive collagen XVII in keratinocytes, but prevented its secretion, thus causing lack of the protein in the skin. The data suggest that different COL17A1 mutations and their combinations can result in a spectrum of biologic and clinical phenotypes of not only generalized atrophic benign epidermolysis bullosa, but also localized junctional epidermolysis bullosa. ***Key words: basement membrane/bullous pemphigoid antigen/collagen/hemidesmosome. J Invest Dermatol 111:528–533, 1998***

Collagen XVII, or 180 kDa bullous pemphigoid antigen, is a structural hemidesmosome component (Nishizawa *et al*, 1993). It is a type II transmembrane molecule with a globular cytoplasmic domain and a large collagenous extracellular domain (Giudice *et al*, 1992; Li *et al*, 1993; Hirako *et al*, 1996; Gatalica *et al*, 1997). As a transmembrane component, it presumably maintains the linkage between intracellular and extracellular structures and anchors the keratinocytes to the basement membrane (Borradori *et al*, 1997). This concept is supported by pathologic conditions. In bullous autoimmune diseases collagen XVII autoantibodies are associated with diminished epidermal–dermal cohesion (Liu *et al*, 1993; Balding *et al*, 1996; Zillikens *et al*, 1997), and in certain junctional epidermolysis bullosa (JEB) subtypes expression of collagen XVII is abnormal (Jonkman *et al*, 1995).

JEB refers to a group of heritable disorders with features of skin fragility, ultrastructurally abnormal hemidesmosomes (McMillan *et al*, 1998), and separation of the epidermis from the basement membrane. JEB includes several subtypes, varying from lethal Herlitz JEB to a mild acral subtype (Gedde-Dahl and Anton-Lamprecht, 1990; Bruckner-Tuderman, 1993). Generalized atrophic benign epidermolysis bullosa (GABEB) represents a subtype with generalized blistering, skin atrophy, alopecia of the scalp, scarce eyelashes, eyebrows, and secondary sexual hair, as well as nail dystrophy and dental anomalies (Hashimoto *et al*, 1976; Hintner and Wolff, 1982). A dozen or so COL17A1 gene mutations have been disclosed in GABEB (McGrath *et al*, 1995, 1996a, b; Darling *et al*, 1997, 1998a, b; Chavanas *et al*, 1997; Gatalica *et al*, 1997; Jonkman *et al*, 1997; Schumann *et al*, 1997) and one mutation in localized acral JEB (Schumann *et al*, 1997). Most of these were homozygous or heterozygous mutations leading to a premature termination codon (PTC) and absence of collagen XVII in the skin. In this study, we identified novel COL17A1 mutations in JEB. Three deletions resulted in PTC, whereas two heterozygous point mutations led to different biologic and clinical phenotypes.

MATERIALS AND METHODS

Patients

Patient 1 The proband, a 75 y old German male, was the fifth child of healthy nonconsanguineous parents. He never had had children and had no living relatives. Two of his older brothers had had similar skin blistering, and two other brothers had died in infancy of "lethal epidermolysis bullosa." The proband had generalized blisters since birth, and affection of the face and the oral mucosa was common. The bullae healed without scars, but with some atrophy and hyperpigmentation. Dystrophy of all nails and dental problems, anamnestically caries, developed in childhood. The proband never had axillary or genital hair, but the scalp hair remained normal. At the age of 75, he had gray hair with male pattern androgenetic alopecia. Thus, he presented with characteristic features of GABEB, except for the normal scalp hair. Electron microscopy showed junctional blistering and hypoplastic hemidesmosomes.

Manuscript received March 4, 1998; revised April 8, 1998; accepted for publication May 18, 1998.

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Abbreviations: GABEB, generalized atrophic benign epidermolysis bullosa; IF, immunofluorescence; JEB, junctional epidermolysis bullosa; PTC, premature termination codon.

Patient 2 The proband, a 6 y old Turkish female, is an offspring of a consanguineous union. No other family member had a similar skin disorder. The proband had fragility of the skin and the mucous membranes since birth, with the extremities and the face being most often affected. The blisters healed without scarring, but were accompanied by dystrophy and loss of most nails. Affection of the scalp led to sparse hair and occipital alopecia. The teeth were normal. Electron microscopy showed junctional blistering and severely hypoplastic hemidesmosomes.

Patient 3 The proband, a 12 y old female, is the younger child of nonconsanguineous Polish parents. The family history was negative for skin diseases. At birth, the proband presented with blisters of the arms and legs. Later, generalized blistering continued, with the extremities, head, face, and oral mucosa being strongly affected. Healing took place without scars, but with skin atrophy and hyperpigmentation. The teeth showed fragile enamel and tendency to dental caries. Nail dystrophy and patchy alopecia of the scalp developed early, leading to a total loss of hair by the age of 12 y. Thus, the proband exhibited the GABEB phenotype with extensive alopecia.

Patient 4 The 34 y old male proband is an offspring of healthy nonconsanguineous Polish parents. No other family member had a related skin disorder. The proband had nonscarring blistering since birth, initially generalized but later localized to the distal extremities. Along the course of the disease, a slight hyperpigmentation of the skin and dystrophy of the toe nails developed. The patient had no dental anomalies, yet tendency to dental caries. Remarkably, the scalp and body hair were completely normal. Based on the clinical presentation, he was initially thought to have epidermolysis bullosa simplex, until antigen mapping revealed junctional splitting and abnormal expression of collagen XVII in the skin.

Mutation detection Genomic DNA was isolated from peripheral blood using the Easy-DNA Kit (Invitrogen, Leek, The Netherlands). Polymerase chain reaction (PCR) amplification of COL17A1 exons 8, 18, 37, 43, and 51 was performed as described by Gatalica *et al* (1997; GenBank No. M91669). The size of the PCR product of exon 8 was 219 bp; exon 18, 384 bp; exon 37, 261 bp; exon 43, 340 bp; and exon 51, 374 bp. Heteroduplex analysis was according to Ganguly *et al* (1993). Heteroduplex forming PCR products were sequenced by an automated sequencer (Genome Express, Grenoble, France).

Northern blotting Northern blotting with keratinocyte mRNA isolated with Oligotex Direct mRNA Minikit (QIAGEN, Hilden, Germany) was performed essentially as described (Schumann *et al*, 1997). For hybridization, a collagen XVII cDNA (Gatalica *et al*, 1997) was labeled with digoxigenin using the digoxigenin DNA Labeling Kit (Boehringer, Mannheim, Germany). The detection was with alkaline phosphatase-labeled digoxigenin-antibodies and CDP-Star substrate (Boehringer).

Keratinocyte cultures Control and JEB keratinocytes were obtained by trypsinization of skin biopsies and cultured in SFM medium (Gibco, Grand Island, NY). Prior to the assays, the cells were grown for 48 h in the presence of 50 μg ascorbic acid per ml to allow for hydroxylation of prolyl residues and correct folding of the collagen (Prockop and Kivirikko, 1995).

Antibodies and immunodetection of proteins The monoclonal antibodies 1A8C to the intra- and 1D1 to the extracellular domain of collagen XVII (Kitajima *et al*, 1992; Nishizawa *et al*, 1993) were used for immunofluorescence (IF) staining of skin cryosections or cultured keratinocytes. A pemphigoid autoantiserum recognizing the NC16a domain of collagen XVII was used for immunoblotting (Schumann *et al*, 1997). For antigen mapping (Hintner *et al*, 1981) the following antibodies were employed: cytokeratins (Dako, Hamburg, Germany), BP230 (a high titer autoantiserum), α6 and β4 integrins (Gibco Life Technologies, Eggenstein, Germany), collagen IV (Dako), laminin 5 (a kind gift from Dr. R. Burgeson, CBRC, Harvard Medical School, Cambridge, MA), and collagen VII (Bruckner-Tuderman *et al*, 1995). For immunoblotting, keratinocytes were extracted with a buffer containing 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4, 1% NP-40, and proteinase inhibitors (Sonnenberg *et al*, 1991; Schumann *et al*, 1997), and the proteins were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 4.5–15% polyacrylamide gradients.

RESULTS

Identification and verification of mutations Mutation screening included PCR amplification of COL17A1 exons directly from genomic DNA (Gatalica *et al*, 1997). In family 1, exon 8 was affected; in family 2, exon 43; in family 3; exons 18 and 37; and in family 4, exon 51. Heteroduplex formation of the patients' DNA mixed with control DNA indicated homozygosity of patients 1 and 2, and heteroduplex formation by the patient's DNA alone indicated heterozygosity of patients 3 and 4 (**Fig 1**). Patient 1 was homozygous for a 520delAG deletion that led to a frameshift and an adjacent PTC. Patient 2 was homozygous for the deletion 2965delG that caused a PTC 334 bp downstream. Verification of this mutation by *Bst*NI endonuclease digestion showed that each parent was heterozygous. Patient 3 was compound heterozygous for a 1721G→A transition in exon 18 and for a 2666delTT deletion in exon 37 that led to a PTC 328 bp downstream. The G→A transition caused a Glu for Gly substitution at amino acid position 539 and was designated G539E. Verification by *Alu*I endonuclease digestion demonstrated that the mother was heterozygous. In contrast, the mutation was not found in 150 normal chromosomes, indicating that it is not a neutral polymorphism. The father was heterozygous for 2666delTT, but the unaffected brother carried neither mutation. Patient 4 was compound heterozygous for a 3781C→T transition designated R1226X (McGrath *et al*, 1995; Jonkman *et al*, 1997; Schumann *et al*, 1997). Verification with *Taq*I digestion demonstrated that his father and sister were heterozygous for this mutation. Despite PCR amplification of all 56 COL17A1 exons, multiple heteroduplex analyses and extensive didesoxynucleotide sequencing failed to reveal the maternal mutation of this proband.

Expression of COL17A1 mRNA Northern blots with normal keratinocyte mRNA showed a strong 6 kb collagen XVII mRNA signal. In contrast, mRNA from patient 1's keratinocytes produced a very weak signal, suggesting nonsense-mediated mRNA decay in these cells (**Fig 2**). Sufficient mRNA from patients 2, 3, and 4 was not available for the analysis.

Collagen XVII in the skin IF staining with antibodies 1A8C and 1D1 was negative in the skin of patients 1, 2, and 3 (**Fig 3*a***, ***c***, ***e***). 1A8C produced a weak staining within basal keratinocytes of patient 4 (**Fig 3*g***). In spontaneously blistered skin of all patients, the antibodies to cytokeratins, BP230, and α6 and β4 integrins stained the blister roof, and the antibodies to laminin 5, collagens IV, and VII stained the blister floor (**Fig 3*b***, ***d***, ***f***, ***h***). This staining pattern thus confirmed junctional tissue separation in all four patients.

Synthesis of collagen XVII *in vitro* IF staining of control keratinocytes with collagen XVII antibodies showed a positive reaction in the rough endoplasmatic reticulum, and in the cell periphery corresponding to the transmembrane location of collagen XVII (**Fig 4*a***). In contrast, the staining of patient 1's keratinocytes was negative (**Fig 4*b***). Staining of patient 3's keratinocytes with the antibody to the intracellular (**Fig 4*c***) and extracellular (**Fig 4*d***) domain of collagen XVII revealed a positive intracellular IF signal. No membrane-bound staining was observed in these cells, indicating intracellular accumulation and degradation of the mutant polypeptides. Keratinocytes of patients 2 and 4 were not available for the studies.

Immunoblotting results were consistent with the IF staining. Keratinocytes were cultured in the presence of ascorbic acid to stabilize newly synthesized collagens (Prockop and Kivirikko, 1995) and immunoblotted with polyclonal collagen XVII antibodies. The 180 kDa α1(XVII)-chain was found in controls, however, and not in extracts of patients 1, 3, and 4 (**Fig 4*e***). This observation indicates that the nascent mutant α1(XVII) chains in the cells of patient 3 (see **Fig 4*c***, ***d***) were degraded intracellularly by default mechanisms. Keratinocytes from patient 2 were not available for these studies.

DISCUSSION

Here we report novel COL17A1 gene mutations associated with JEB. All but one (Schumann *et al*, 1997) of the previously described COL17A1 defects (McGrath *et al*, 1995, 1996a, b; Darling *et al*, 1997, 1998a, b; Chavanas *et al*, 1997; Gatalica *et al*, 1997; Jonkman *et al*, 1997) were associated with the GABEB phenotype, including generalized blistering, skin atrophy, nail dystrophy, and, the clinical hallmark of this JEB subtype, extensive nonscarring alopecia. Interestingly, the three GABEB patients of this study showed a variable degree of alopecia. Patient 1, now 75 y old, still had normal scalp hair. The 6 y old patient 2 exhibited an intermediate, and patient 3 a drastic phenotype with complete baldness at the age of 12 y.

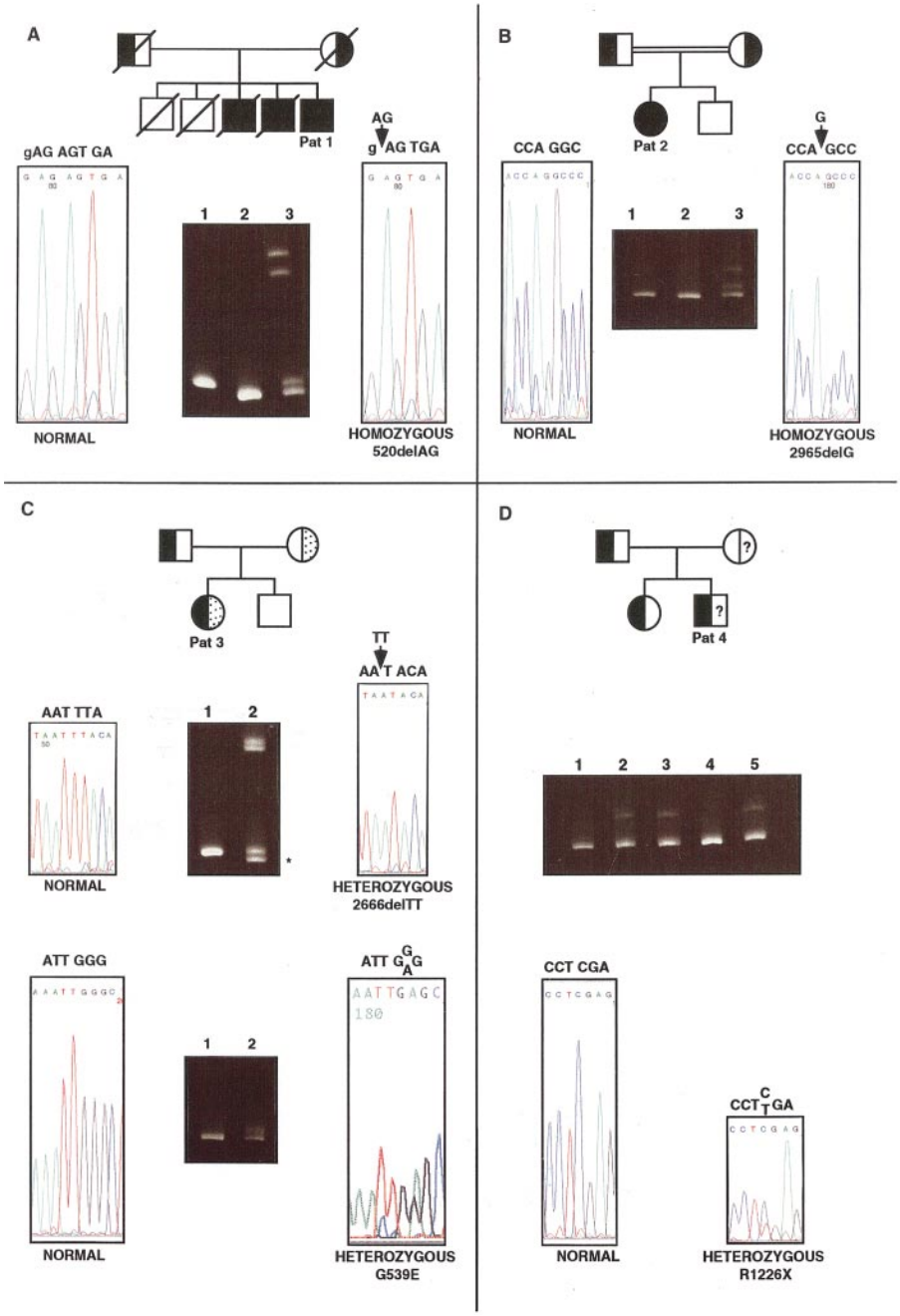

Figure 1. **Pedigrees of the probands and mutations in the COL17A1 gene.** Pedigrees demonstrate recessive inheritance of the mutations. (*A*) family 1; (*B*) family 2; (*C*) family 3; (*D*) family 4. (*A*) Heteroduplex bands were detected on CSGE gels of PCR product spanning exon 8 (*middle panel*). Control DNA (*lane 1*) and the patient's DNA (*lane 2*) showed homoduplex bands when tested alone. The faster migration of the patient's homoduplex band suggested a deletion in this fragment. Heteroduplex bands were seen in mixed samples of the patient's and control DNA (*lane 3*). Didesoxynucleotide sequencing of the patient's DNA revealed a homozygous 2 bp deletion in exon 8, designated 520delAG (*right panel*), whereas the deletion was absent from a control (*left panel*). (*B*) CSGE analysis of PCR product spanning exon 43 (*middle panel*) revealed a homoduplex band in control DNA (*lane 1*) and the patient's DNA (*lane 2*), but heteroduplex bands when the samples were mixed with each other (*lane 3*). Direct didesoxynucleotide sequencing of the patients' PCR products revealed a homozygous 1 bp deletion of nucleotide G in exon 43, designated 2965delG (*right panel*), which was absent from a control (*left panel*). (*C*) CSGE analysis of the PCR products spanning exons 18 and 37 revealed heteroduplex bands with the patient's DNA, indicating compound heterozygosity of the proband (*middle panels*). The upper middle panel shows CSGE analysis of PCR product spanning exon 37 (paternal mutation); control DNA (*lane 1*) showed a homoduplex band and the patient's DNA (*lane 2*) homo- and heteroduplex bands. The lower homoduplex band (*asterisk*) was excised, eluted, reamplified, and subjected to didesoxynucleotide sequencing that revealed a heterozygous 2 bp deletion in exon 37, designated 2666delTT (*right panel*), which was absent from a control (*left panel*). This heterozygous deletion was also found in the father's DNA. The lower middle panel shows CSGE analysis of PCR product spanning exon 18 (maternal mutation); control DNA (*lane 1*) showed a homoduplex band and the patient's DNA (*lane 2*) heteroduplex bands. Direct didesoxynucleotide sequencing of the patients' PCR products revealed a heterozygous 1721G→A transition in exon 18, designated G539E (*right panel*), which was absent from a control (*left panel*). This heterozygous mutation was also found in the mother's DNA. In the pedigree, the black areas indicate the paternal and the dotted areas the maternal allele. (*D*) Identification of the paternal mutation. CSGE analysis of PCR product spanning exon 51 (*middle panel*) revealed a homoduplex band in control (*lane 1*) and the mother's DNA (*lane 4*). In contrast, heteroduplex bands were seen with the patient's (*lane 2*), the father's (*lane 3*), and the sister's DNA (*lane 5*). Direct didesoxynucleotide sequencing of the patient's PCR products revealed a heterozygous 3781C→T transition in exon 51, designated R1226X (*right panel*). The mutation was also found in the unaffected father and sister, but not in a control or the mother (*left panel*). Extensive mutation screening did not reveal other changes in the COL17A1 gene and therefore the maternal mutation remained elusive.

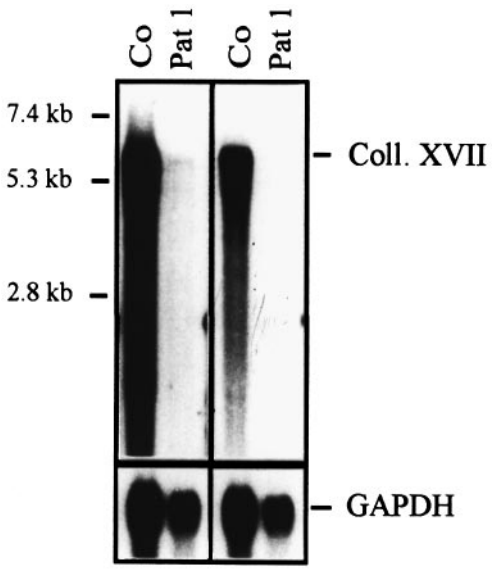

Figure 2. Northern blotting of collagen XVII mRNA from normal and GABEB keratinocytes. A strong signal of the 6 kb collagen XVII mRNA was found in control keratinocytes (Co). In contrast, collagen XVII mRNA isolated from the patient 1's keratinocytes (Pat 1) produced a very weak signal, suggesting nonsense-mediated mRNA decay in these cells. The panels were intentionally overexposed to demonstrate the presence of the weak collagen XVII mRNA band in the patient's cells. A digoxigenin-labeled GAPDH cDNA was used as a reference probe to correct for differences in RNA loading.

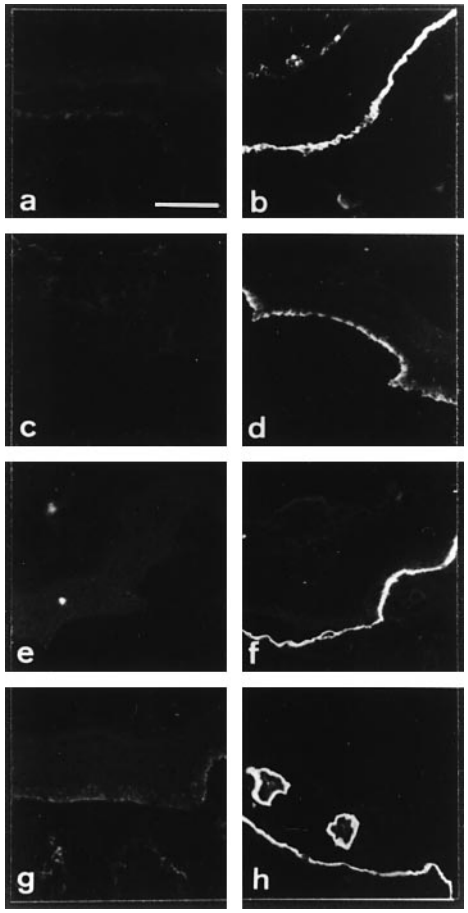

Figure 3. IF staining of the patients' skin with antibodies to cutaneous basement membrane components. The biopsies and antibodies used were: (*a*) patient 1, 1A8C; (*b*) patient 1, laminin 5; (*c*) patient 2, 1A8C; (*d*) patient 2, integrin α6; (*e*) patient 3, 1A8C; (*f*) patient 3, collagen VII; (*g*) patient 4, 1A8C; (*h*) patient 4, collagen VII. In (*g*), a weak reactivity with the antibody 1A8C was observed in the basal keratinocytes of the detached epidermis. The stainings with antibodies to other basement membrane proteins were normal. In blistered skin of patient 2, antibodies to integrin α6 localized to the blister roof (*d*). Antibodies to collagen VII stained the blister base in separated skin of patients 3 (*f*) and 4 (*h*). The staining patterns were consistent with junctional blistering. *Scale bar*: 40 μm.

Patients 1 and 2 were homozygous for a PTC and lacked collagen XVII in the skin. The biologic phenotype was in concert with the observation that most mutations resulting in PTC cause nonsense-mediated mRNA decay and lack of the synthesis of the corresponding protein (Cui *et al*, 1995). Patient 3 was compound heterozygous for a missense (G539E) and a nonsense (2666delTT) mutation. Unexpectedly, this combination also led to lack of collagen XVII in the skin. When cultured, the proband's keratinocytes synthesized and retained intracellularly immunoreactive polypeptides, but no deposition of collagen XVII at the plasma membrane was observed. The nonsense mutation is likely to result in accelerated mRNA decay (Darling *et al*, 1998b) and therefore the proband can be regarded as hemizygous for the G539E substitution at the mRNA and protein level. The Gly substitution by Glu in the NC16a domain seems to destabilize the nascent polypeptides and cause retention of mutant molecules in the cell and subsequent degradation through default pathways, a phenomenon coined "protein suicide" of mutant trimeric collagens (Prockop and Kivirikko, 1995). The mutant α1(XVII) chains were only seen by IF in keratinocytes with a high level of expression *in vitro*, but they were not sufficiently stable to resist the extraction and immunoblotting procedures. Neither were they visible by IF in the epidermis where the rate of synthesis is low. One possible explanation for the extensive intracellular retention and degradation is that the G539E substitution occurred within a functionally essential sequence of the NC16a domain, adjacent to the transmembrane domain. This segment contains the immunodominant epitopes in autoimmune blistering disorders (Zillikens *et al*, 1997), and binding of the autoantibodies to the epitopes has been shown to lead to perturbation of collagen XVII functions (Liu *et al*, 1993).

Two other missense mutations in COL17A1 have been reported. In a compound heterozygous GABEB patient with a less severe phenotype, McGrath *et al* (1996b) disclosed a G627V substitution within the COL15 domain, in combination with an internal duplication that led to a PTC. Similar to our patient 3, the proband lacked collagen XVII in the skin. The authors regarded G627V as dominant, because the proband's offspring who had inherited this mutation presented with dental anomalies, but not with skin fragility. In the present family 3, the mother heterozygous for G539E was phenotypically normal and without dental problems. Interestingly, both glycine substitutions G627V and G539E in the NC16a domain were associated with GABEB phenotypes of different severity. In contrast, a homozygous R1303Q substitution at the C-terminus of collagen XVII led to JEB *localisata* (Schumann *et al*, 1997). This mutation did not influence secretion of collagen XVII, but possibly created a novel transglutaminase cross-linking site, because large collagen XVII aggregates were found in the epidermis.

Our patient 4 presented with JEB *localisata* and normal hair, despite a COL17A1 nonsense mutation. He was heterozygous for R1226X previously reported in GABEB families from the U.K., Holland, and Germany (McGrath *et al*, 1995; Jonkman *et al*, 1997; Schumann *et al*, 1997). This mutation may represent a mutational hot spot similar to one in the LAMB3 gene associated with JEB Herlitz (Kivirikko *et al*, 1996). Alternatively, the mutation may have a common founder in Central Europe, as observed for the COL17A1 mutation 4003delTC that represents a common ancestral allele in Austrian GABEB families (Darling *et al*, 1998a).

The phenotypic heterogeneity of COL17A1 defects is likely to be greater than previously expected. Mutation combinations can lead to different JEB phenotypes, and not all nonsense mutations are associated with GABEB and extensive alopecia, but can result in milder clinical presentation. Mutation screening and biologic and protein chemical

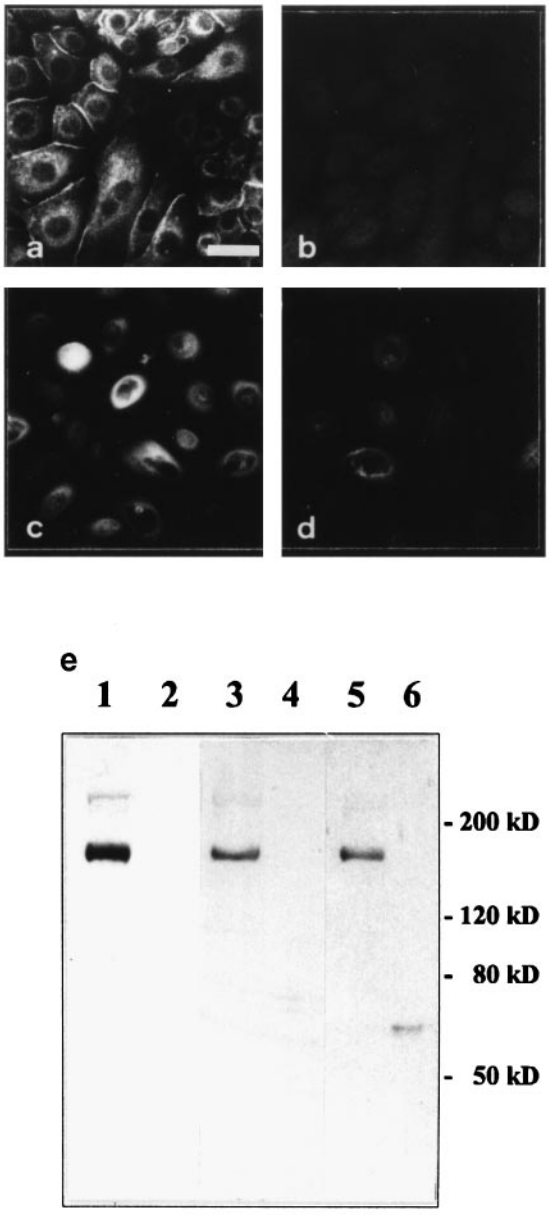

Figure 4. Immunodetection of collagen XVII in keratinocytes *in vitro*. (*a–d*) IF staining of GABEB keratinocytes with domain-specific antibodies to collagen XVII. In control cells, the antibody 1D1 to the extracellular domain showed a positive reaction in the rough endoplasmatic reticulum and in the cell periphery, corresponding to the transmembrane localization (*a*). The antibody produced a negative reaction in the keratinocytes of patient 1 (*b*). Staining of keratinocytes of patient 3 with the antibody 1A8C to the intracellular domain revealed a positive diffuse intracellular fluorescence (*c*), and with the antibody 1D1 to the extracellular domain a diffuse weak reaction (*d*). *Scale bar*: 10 μm. (*e*) Immunoblotting of keratinocyte extracts with polyclonal antibodies to the NC16a domain. Normal controls contained the 180 kDa α1(XVII)-chain (*lanes 1*, *3*, *5*). In contrast, cell extracts of patient 1 (*lane 2*), patient 3 (*lane 4*), and patient 4 (*lane 6*) lacked the band, indicating absence of stable collagen XVII in these cells. The 65 kDa band in *lane 6* was occasionally seen with the human autoantiserum used, but its identity has not been characterized. The migration positions of molecular weight markers are indicated on the right.

analyses of further JEB families will reveal the genotype-phenotype correlations and lay a basis for novel gene therapeutic approaches for JEB.

The authors thank Ms. M. Schubert and Ms. A. Wissel for superb technical assistance. This investigation was supported by grants Br 1475/1–2 and SFB 293 Münster/B3 to L.B.-T. from the Deutsche Forschungsgemeinschaft (DFG) and by the EU contract BMH4-CT97–2062.

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